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The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment by human activity have also been found to act as estrogen mimics and alter reproductive function and development. The goals of this project are to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. Towards this end, we have expressed and purified the ligand binding domain of the estrogen receptor, and studied complexes with estradiol, the natural ligand, and tamoxifen, a partial antiestrogen in use as a breast cancer therapeutic using high-resolution heteronuclear NMR spectrosocpy.

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Deborah Smith 6/30/00

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Annual Report for Breast Cancer Therapeutics, Environmental Estrogens, and the Estrogen Receptor (ER): Characterization of the Diverse Ligand Binding Properties of the ER

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INTRODUCTION

The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment by human activity have also been found to act as estrogen mimics and alter reproductive function and development. The estrogenic behavior of these compounds has proven difficult to predict from their structures; many of these hormone mimics bear little structural resemblance to natural estrogens. The molecular level details of the conformational changes that allow the ER to tightly bind a diverse array of compounds and result in diverse patterns of gene activation are not understood. The goals of this project are to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. A complete structural understanding of how various ligands interact with the ER, and are able to elicit different responses, will assist in identifying compounds with therapeutic benefit for treating breast cancer. In addition, our studies will contribute to understanding the effects environmental estrogens have on breast cancer, and in identifying estrogenic activity in compounds before they are introduced into the environment.

BODY

The large numbers of compounds which bind the ER will require the use of an innovative and rapid approach. We are investigating ligand binding to the ER using multidimensional nuclear magnetic resonance spectroscopy (NMR) studies of complexes of these compounds with the ligand binding domain (LBD) of the ER. The ER-LBD is uniformly isotopically labeled with ^{13}C and ^{15}N and ligand binding will be followed by acquiring heteronuclear single quantum coherance (HSQC) spectra, which are exquisitely sensitive to molecular conformation. Also, we will study the structural effects of several point mutations isolated from breast cancer cell lines. We will investigate the ligand binding differences between ERα and ERβ, the two natural variants of the ER. These differences may play an important role in the tissue specific activity of partial antiestrogens. Analysis of our NMR data will be significantly aided by the present availability of the crystal structures of the ER bound to an estrogen and an antiestrogen. The use of NMR spectroscopy as an efficient tool for screening for compounds with estrogenic or antiestrogenic activity, or with activity specific to either ERα or ERβ will be explored.

Progress towards the tasks in the statement of work are as follows:

Technical Objective 1:

Prepare and purify unlabeled LBD-ER_	2 Months
Identify solution conditions suitable for NMR experiments	1 Months
Collect and evaluate preliminary homonuclear NMR data	1 Month

The tasks associated with technical objective 1 have been completely achieved. The LBD-ER was prepared by expression in BL21(DE3) *E. coli* by induction with IPTG from a pET plasmid. Complete purification was achieved using an estradiol affinity column that afforded protein of high purity and activity in one step. In addition, the purified protein is highly stable to proteolysis, a problem that rendered protein purified by other strategies inactive within a few days of preparation. A complete buffer screen revealed optiminal buffer conditions (20mM d-Tris, 200mM NaCl, 1mM d-DTT, 0.02% sodium azide) that allowed us to prepare samples with concentrations of up to 500 μM. Homonuclear NMR data obtained on these samples revealed features consistent with a protein of the anticipated molecular weight. The optimum temperature for data collection was determined to be 30°C. The anticipated molecular weight of the estradiol bound complex was confirmed by light scattering data.

Technical Objective 2:

Prepare and purify ligand complexes of ^{15}N-labeled ER from minimal growths	2 Months
Collect and evaluate ^{15}N-^{1}H HSQC NMR data at 500 MHz	1 Months
Collect and evaluate ^{15}N-^{1}H HSQC NMR data at 600 MHz	1 Month
Prepare and purify ligand complexes of ^{13}C,^{15}N-labeled ER from minimal growths	2 Months
Collect and evaluate ^{13}C and ^{15}N-^{1}H HSQC NMR data at 500 MHz	1 Months
Collect and evaluate ^{13}C and ^{15}N-^{1}H HSQC NMR data at 600 MHz	1 Month
Prepare and purify ligand complexes of isotopically labeled ER from minimal growths	3-9 Months
Collect and evaluate ^{13}C and ^{15}N-^{1}H HSQC NMR data of complexes at optimum NMR conditions	6-12 Months
Investigate deuteration strategies	6-9 Months

The tasks associated with technical objective 2 have been completely achieved. Growth of uniformly ^{15}N labeled protein progressed smoothly based on the expression and purification scheme developed for unlabeled protein. ^{15}N-^{1}H HSQC NMR data were collected at 500 and 600 MHz. Significant improvement of data quality was observed at higher field strengths and with the addition of TROSY pulse sequences. Based on these data, we determined that data should be collected at as high of a field strength as possible; towards this end we are pursing collaborations with researchers with access to an 800 MHz spectrometer. In addition, TROSY-based pulse sequences will be used exclusively to study this system given the large enhancement. A tamoxifen-bound complex was prepared to compare tamoxifen to estradiol-bound forms of the protein. Significant chemical shift differences were noted between these two complexes, as anticipated, illustrating the validity of our strategy.

In order to interpret the chemical shifts in the context of available structures, resonance assignments need to be completed. Towards this end, uniformly ^{13}C, ^{15}N and ^{2}H labeled protein has been prepared. The ^{13}C and ^{15}N labels will allow assignment of the backbone resonances via their direct scalar couplings. Complete deuteration is employed to further reduce linewidths by eliminating the dominant mechanism of line broadening, relaxation via ^{1}H-^{1}H dipolar couplings. To achieve this result, growth in D_2O has been optimized. Initial efforts aimed at obtaining triple resonance data for resonance assignments indicate that further optimization, either through preparation of higher concentration samples or data collection at higher field, will be necessary.

In addition to the work described above, CD denaturation studies have been conducted on the estradiol and tamoxifen complexes. These data have allowed us to compare the thermodynamic stabilities of these complexes.

Future work on this project will be focused on Technical objectives 3 and 4. If it is not feasible to obtain resonance assignments using the fully labeled protein, selective labeling strategies allowing uniform incorporation of only one amino acid type or selective labeling of the C-terminus will be employed.

KEY RESEARCH ACCOMPLISHMENTS

- Highly purified LBD-ER has been prepared.
- Conditions for NMR data collection have been optimized.
- ^{15}N-^{1}H HSQC NMR data on estradiol and tamoxifen complexes has been obtained.
- Uniformly ^{13}C, ^{15}N and ^{2}H labeled protein has been prepared.
- The process of resonance assignment has been initiated.

REPORTABLE OUTCOMES

Posters presented: The work described in this progress report has been presented at several meetings as either a talk or poster. These include (1) The University of Colorado Annual Retreat (Winter Park, Colorado) (2) Environmental Hormones: Past, Present, Future (Tulane University, New Orleans, LA) (3) The University of Colorado Biophysics Supergroup Meeting and (4) The EPA-STAR Award Annual Meeting (Washington, DC).

Graduate Student Training: One graduate student, Dana Warn, has been trained on this research project. She has been primarily responsible for protein expression and purification protocols, and NMR spectroscopy. Her stipend and tuition has been provided through an EPA-STAR fellowship.

Undergraduate Training: Andrea Wismann, an undergraduate at the University of Colorado, Boulder, conducted independent research on this project from August, 1999 through June 2000. Through this research experience, she learned protein expression and purification strategies, as well as circular dichroism spectropolarimetry.

CONCLUSIONS

In the first year of funding, good progress has been made towards the stated goals. Highly purified, stable samples have been obtained. Complexes of LBD-ER with estradiol and the partial antiestrogen tamoxifen have been prepared and characterized by high resolution NMR spectroscopy. Preliminary experiments aimed at complete resonance assignment have been

initiated. Future work towards technical objectives 3 &4 has been undertaken. In addition, alternate strategies for obtaining assignment information are being developed.

REFERENCES

No references are included in this report.

APPENDICES

No appendices are attached.